Decreased Susceptibility of *Staphylococcus aureus* Small-Colony Variants toward Human Antimicrobial Peptides

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Staphylococcus aureus is a frequent resident of human nose and skin in many individuals, but it is also able to cause a variety of serious infections including those of the skin and soft tissue. There is increasing evidence that particularly persistent, relapsing, and difficult-to-treat infections caused by *S. aureus* are associated with the formation of the small-colony variant (SCV) phenotype. The aim of this study was to investigate the hypothesis that (i) skin-derived antimicrobial peptides (AMPs) exhibit a reduced activity against SCVs and (ii) that switching into the SCV phenotype may endow *S. aureus* with a decreased susceptibility toward the killing activity of human stratum corneum. Here, we show that clinically derived *S. aureus* SCVs are less susceptible to the bactericidal activity of different human skin-derived AMPs as compared with their isogenic corresponding wild-type strains. Similarly, a *S. aureus hemB* mutant displaying the SCV phenotype was less susceptible to the antimicrobial activity of AMPs than its *hemB*-complemented mutant. These findings were accompanied by a higher resistance of SCVs to the killing activity of human stratum corneum. Switching into the SCV phenotype may help *S. aureus* to subvert cutaneous innate defense, thus contributing to the establishment and persistence of infection.

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INTRODUCTION

The Gram-positive opportunistic bacterium *Staphylococcus aureus* is a frequent resident of human skin and nose microbiota in many individuals (Wos-Oxley *et al.*, 2010), but it also has a major impact as a causative agent of a variety of serious infections including those of the skin and soft tissue (Iwatsuki *et al.*, 2006; Miller and Kaplan, 2009). There is increasing evidence that particularly persistent, relapsing, and difficult-to-treat infections caused by *S. aureus* are associated with the formation of the small-colony variant (SCV) phenotype (Proctor *et al.*, 2006; Vaudaux *et al.*, 2006; von Eiff and Becker, 2007). SCVs generally have a longer generation time and are named because of their slow growth leading to small colonies on agar plates. As SCVs often need several days to become visible on agar plates, they can be easily missed by routine microbial diagnostic procedures (Proctor *et al.*, 2006; Vaudaux *et al.*, 2006). Being internalized in the host cell environment, *S. aureus* is able to switch from the wild-type phenotype to a SCV phenotype, thus making it possible to escape host defense responses and to spread the infection (Tuchscherr *et al.*, 2011; von Eiff *et al.*, 2001). The switch to an SCV phenotype is associated with complex physiological and metabolic changes mainly based on defects in electron transport or in biosynthesis of thymidine (Proctor *et al.*, 2006; Kriegeskorte *et al.*, 2011). The phenotype switching enables *S. aureus* to hide inside host cells, leading to escape from immune responses and those antibiotics without intracellular activity (von Eiff *et al.*, 2006; Garcia *et al.*, 2013).

SCVs have been associated with various chronic, recurrent, and persistent infections including soft-tissue and skin infections (von Eiff *et al.*, 2006; Melter and Radojevic, 2010). As the epidermis is the first barrier that *S. aureus* has to overcome in order to enter and persist in the host, we hypothesize that SCVs may subvert cutaneous defense by a decreased susceptibility toward skin-derived AMPs.

RESULTS AND DISCUSSION

The aim of this study was to investigate the hypothesis that SCVs display a higher resistance toward the action of skin-derived antimicrobial peptides (AMPs). To this end, we analyzed the activity of the skin-derived AMPs human beta-defensin (hBD)-2 and -3, RNase 7, and LL-37 against various SCVs. We choose these AMPs because they are all major skin-derived AMPs (Harder *et al.*, 2007; Gallo and Hooper, 2012), and they are all present in the stratum corneum, as demonstrated by immunohistochemistry (Figure 1). In particular, RNase 7 and hBD-3 have been reported to be principle AMPs

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Abbreviations: AMP, antimicrobial peptide; hBD, human beta-defensin; SCV, small-colony variant

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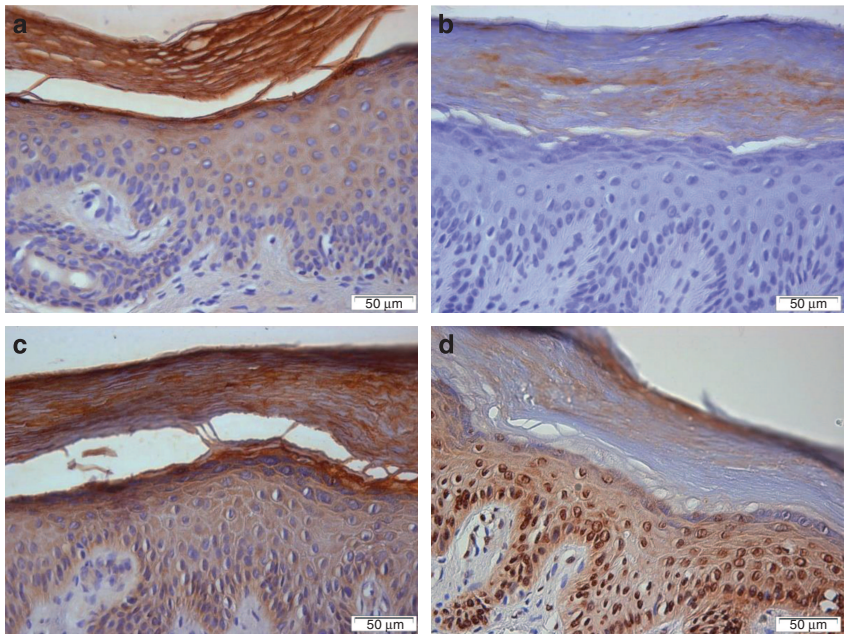

Figure 1. Immunostaining of RNase 7, human beta-defensin (hBD)-2, hBD-3, and LL-37 in the stratum corneum. To demonstrate the presence of the investigated antimicrobial peptides (AMPs) in the stratum corneum, we performed immunohistochemical staining of the AMPs using human skin biopsies derived from the soles of the feet. RNase 7 (**a**), hBD-2 (**b**), hBD-3 (**c**), and LL-37 (**d**) all showed staining in the stratum corneum. Bars = 50 µm.

Table 1. LD_{90} (µg ml^{-1})[1] of the AMPs RNase 7, hBD-2, hBD-3, and LL-37 against SCV and corresponding wild-type strains OM1, A22216, and OM299, as well as against a *S. aureus hemB* mutant and its *hemB*-complemented mutant

	OM1		A22216		OM299		*hemB*	
	Wild-type	SCV	Wild-type	SCV	Wild-type	SCV	Complemented mutant	Mutant
RNase 7	2.5–5	>40	2.5–5	>40	5–10	>40	2.5–5	>40
hBD-2	10–20	>40	10–20	>40	5–10	>40	>40 (10–20)[2]	>40 (>40)[2]
hBD-3	0.6–1.25	2.5–5	0.6–1.25	1.25–2.5	0.6–1.25	1.25–2.5	0.6–1.25	1.25–2.5
LL-37	2.5–5	5–10	2.5–5	10–20	1.25–2.5	10–20	5–10	5–10

Abbreviations: AMPs, antimicrobial peptides; hBD, human beta-defensin; LD, lethal dose; SCV, small-colony variant.
[1]Lethal dose (µg ml^{-1}) to kill 90% of the bacteria.
[2]Lethal dose (µg ml^{-1}) to kill 50% of the bacteria.

released by keratinocytes to control the growth of *S. aureus* (Kisich *et al.*, 2007; Simanski *et al.*, 2010).

We tested these AMPs against three natural SCV isolates recovered from clinical specimens in parallel with their isogenic corresponding wild-type strains. The use of a microdilution assay with different concentrations of AMPs revealed highest activity of hBD-3 against wild-type *S. aureus* followed by LL-37, RNase 7, and hBD-2 (Table 1). In contrast, all SCVs showed a decreased susceptibility toward these AMPs. HBD-3 demonstrated a moderate reduction of its capability to kill SCVs compared with wild-type *S. aureus*. RNase 7 and hBD-2 showed a strongly reduced antimicrobial activity against SCVs. Similarly, LL-37 displayed a decreased activity against all clinical SCVs (Table 1).

It is obvious that hBD-3 killed those isolates that display the SCV phenotype more efficiently than the other AMPs, including its beta-defensin family member hBD-2. It is known that hBD-2 is in general less active against *S. aureus*, and this may be the reason why hBD-2 is also less active against SCVs. It has been suggested that the capacity of hBD-3 to form dimers and its high positive surface charge are responsible for its high activity against S. *aureus* (Schibli *et al.*, 2002). In addition, its special mode of action by interfering with the *S. aureus* cell wall biosynthesis machinery (Sass *et al.*, 2010) may also explain its augmented activity against *S. aureus* and, therefore, also against SCVs. Nevertheless, hBD-3 showed also a decreased activity against all tested SCVs as compared with the corresponding wild-type strains (Table 1).

To further verify the reduced activity of AMPs against SCVs, we used a strain pair representing (i) a genetically defined mutant constructed by interrupting one of the hemin biosynthetic genes, *hemB,* that displays the SCV phenotype, and (ii) its *hemB*-complemented mutant exhibiting the normal phenotype (von Eiff *et al.*, 1997). Except LL-37, all AMPs exhibited

decreased antimicrobial activity against the *hemB* mutant as compared with the *hemB*-complemented mutant (Table 1). Taken together, our data indicate that skin-derived AMPs generally exhibit a limited antimicrobial activity against SCVs.

To further evaluate the functional relevance of these findings, we incubated stratum corneum extract (derived from heel callus) with the different bacterial strains and analyzed the killing activity of the extract. These experiments revealed a significantly reduced activity of the stratum corneum extract against all three clinical SCVs tested, as compared with the corresponding wild-type *S. aureus* strains (Figure 2a–c). In concordance with these findings, the *hemB* mutant displaying the SCV phenotype was less susceptible to the killing activity of the stratum corneum as compared with the *hemB*-complemented strain (Figure 2d).

Our results demonstrate a reduced activity of human AMPs against SCVs. The reduced activity of human skin-derived AMPs against SCVs is accompanied by a decreased capability of the stratum corneum to kill isolates exhibiting the SCV phenotype. Recently, it has been demonstrated that the antimicrobial activity of stratum corneum extract against *S. aureus* was in part mediated by RNase 7 (Simanski *et al.*, 2010). This suggests that the observed antimicrobial activity of the stratum corneum extract against *S. aureus* and *S. aureus* SCVs is mediated by AMPs such as RNase 7. However, we cannot exclude that also other antimicrobial factors in the stratum corneum (e.g., antimicrobial lipids, (Drake *et al.*, 2008)) may contribute to the observed antimicrobial activity of the stratum corneum extract.

The reason why SCVs are endowed with a higher resistance toward the activity of AMPs is not clear. Most human AMPs, including the AMP under investigation used in this study, are cationic and therefore positively charged. It has been suggested that the resistance of SCVs against positively charged antimicrobial agents might be a result of a lower electrochemical gradient across the bacterial cell membrane (Sadowska *et al.*, 2002). However, cationic AMPs do not always exhibit a reduced activity against SCVs. Sadowska *et al.* (2002) tested several cationic AMPs against SCVs, and they found no significant differences in the susceptibility of normal *S. aureus* and SCVs to the bactericidal activity of the frog-derived cationic AMP magainin and the neutrophil-derived alpha-defensin HNP-1. The susceptibility to the frog-derived cationic AMP dermaseptin was even higher for SCVs (Sadowska *et al.*, 2002). In contrast, SCVs were more resistant toward the fish-derived cationic AMP protamine (Sadowska *et al.*, 2002), toward the bovine-derived cationic AMP lactoferricin B (Samuelsen *et al.*, 2005), and toward the rabbit-derived thrombin-induced platelet microbicidal protein (Koo *et al.*, 1996). Uncovering the exact resistance mode of SCVs toward cationic antibiotics and AMPs is a future challenge to develop novel antimicrobial agents with approved activity toward SCVs.

In summary, SCVs are less susceptible to the bactericidal activity of different human skin-derived AMPs, and these findings are associated with a higher resistance to the killing activity of human stratum corneum. Switching into the SCV phenotype may help *S. aureus* subvert cutaneous innate defense, thus contributing to the establishment and persistence of infection.

MATERIALS AND METHODS

Immunohistochemistry

To investigate the presence of AMPs in the stratum corneum, we performed immunohistochemistry using formalin-fixed, paraffin-embedded skin samples derived from the soles of patients ($n=3$) undergoing surgery of benign skin disorders (moles) after having given informed consent (Kiel, AZ A104/06). Immunohistochemistry was performed as described (Harder *et al.*, 2010; Wittersheim *et al.*, 2013) using antibodies against hBD-2 (Peprotech, Hamburg, Germany), hBD-3 (Acris Antibodies, Herford, Germany), LL-37 (Innovagen; Lund, Sweden), and RNase 7 (Köten *et al.*, 2009).

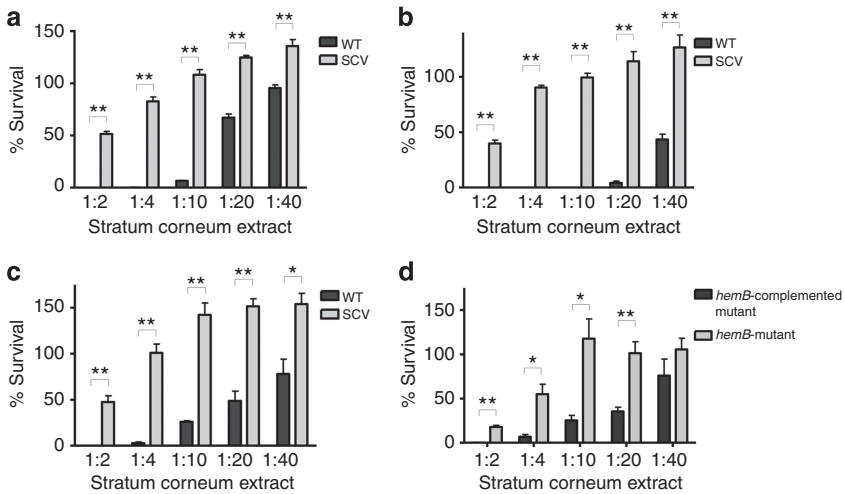

Figure 2. Decreased susceptibility of *S. aureus* small-colony variants (SCVs) to the killing activity of human stratum corneum extract. Various dilutions of stratum corneum extract were tested in a microdilution assay for their capacity to kill various SCVs. Bacteria were incubated together with the indicated dilutions of stratum corneum extract for 3 hours. After 3 hours, serial dilutions were plated on trypticase soy broth agar plates, and colony-forming units were counted after incubation for up to 4 days. The growth control after 3 hours without stratum corneum extract was set as 100%. Bars are means ± SD representative of at least two independent experiments (*$P<0.05$, **$P<0.01$; Student's *t*-test). Shown are the *S. aureus* SCV clinical isolates OM1 (**a**), OM299 (**b**), and A22216 (**c**) and their corresponding isogenic wild types. In addition, a *S. aureus hemB* mutant displaying the SCV phenotype and its *hemB*-complemented mutant were used (**d**).

SCVs of *Staphylococcus aureus*

We used three natural SCV isolates recovered from clinical specimens in parallel with their isogenic corresponding wild-type strains. Genetic relatedness between clinical SCVs and their corresponding parental strains displaying the normal phenotype was determined by pulsed-field gel electrophoresis), as described elsewhere (von Eiff *et al.*, 2001).

Furthermore, a genetically constructed strain pair consisting of a site-directed *S. aureus hemB* mutant and its *hemB*-complemented mutant was included (von Eiff *et al.*, 1997). The *hemB* mutant displays the SCV phenotype and represents a genetically defined mutant constructed by interrupting one of the hemin biosynthetic genes. As a corresponding control, the *hemB*-complemented mutant, which exhibits the normal phenotype, was used (von Eiff *et al.*, 1997).

Antimicrobial activity of AMP and stratum corneum extract toward SCVs

We used a microdilution assay to test the antimicrobial activity of the AMPs hBD-2 and -3, RNase 7, and LL-37 against the SCVs. The microdilution assay was performed by incubating bacteria and serial dilutions of AMPs for 3 hours in sodium phosphate buffer (pH 7.2) containing 1% trypticase soy broth. The remaining bacteria were determined by plating on trypticase soy broth (Sigma, Munich, Germany) agar plates and counting colony-forming units after incubation at 37 °C for up to 4 days. Recombinant hBD-2, hBD-3, and RNase 7 were produced in *E. coli* as described (Harder *et al.*, 2001; Köten *et al.*, 2009; Sahly *et al.*, 2003). Synthetic LL-37 was obtained from Innovagen.

Stratum corneum derived from healthy persons' heel callus was extracted as described (Schröder, 2010) and diafiltered against 10 mM sodium phosphate buffer (pH 7.2). To analyze the antimicrobial activity of the stratum corneum extract against *S. aureus* wild-type and SCV strains, various dilutions of this extract were incubated in a microdilution assay, as described above.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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